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Metabolism of *Mycobacterium leprae* in Macrophages

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The incorporation of ^{14}C-labeled palmitic acid ([U-^{14}C]PA) into the phenolic glycolipid-I (PGL-I) fraction of *Mycobacterium leprae* was studied in a murine macrophage system in vitro. Peritoneal macrophages from Swiss Webster mice were infected with fresh viable or Formalin-killed *M. leprae* harvested from infected footpads of *nu/nu* mice, and [U-^{14}C]PA was added to the culture medium. Labeled glycolipid synthesized by live *M. leprae* was fractionated on a Florisil-silicic acid column and identified as PGL-I by using thin-layer chromatography and localization on a polysulfone membrane with an anti-PGL-I monoclonal antibody. Increased incorporation of [U-^{14}C]PA into the PGL-I fraction was observed in macrophages infected with only live *M. leprae*. Treatment of the infected macrophages with rifampin caused a significant reduction in the incorporation of palmitic acid into PGL-I. These preliminary studies suggest that PGL-I synthesis can be used to quantitate the metabolism of *M. leprae* in macrophages in vitro.

The inability to rapidly quantitate the viability of *Mycobacterium leprae* has vastly impeded progress in leprosy research. To monitor the viability of the noncultivable leprosy bacillus, tedious indirect titrations employing the mouse footpad technique developed by Shepard (21) are required, often taking a year or more to complete (13). Clearly, to more easily screen antileprosy chemotherapeutic regimens or to study host resistance factors in leprosy there is a need to develop alternate and more rapid techniques for determining the viability of *M. leprae*.

M. leprae is a natural intracellular parasite that depends on the internal milieu of the host cell for survival. Within the host cell, *M. leprae* synthesizes large amounts of a characteristic phenolic glycolipid (PGL-I) which has been shown by Brennan and colleagues to be biochemically unique and immunologically specific for *M. leprae* (1, 9, 10). Since mononuclear phagocytes have been shown to serve as important host cells for *M. leprae*, the present study explores the biosynthesis of PGL-I by the leprosy bacillus in tissue cultures of mouse peritoneal macrophages. The in vitro biosynthesis of PGL-I from ^{14}C-acetate by *M. leprae* within schwannoma cell lines has been reported by Mukherjee et al. (16). In our studies PGL-I synthesis was monitored in vitro by measuring the incorporation of radiolabeled palmitic acid (PA) into the extractable PGL-I fraction of *M. leprae* in infected macrophages. The present work describes the methodology developed and reports our preliminary findings on the effects of rifampin on PGL-I synthesis by *M. leprae* in vitro.

MATERIALS AND METHODS

Macrophages. Peritoneal cells (PC) were harvested from normal unstimulated Swiss Webster mice by using methods described previously (12). Briefly, PC harvested in heparinized Hanks balanced salt solution containing 10 U of heparin per ml were washed by centrifugation (150 × *g* for 10 min) and suspended in medium RPMI 1640 (GIBCO Laboratories, Chagrin Falls, Ohio) supplemented with 20% heat-inactivated fetal calf serum (RPMI-20% FCS; Sterile Systems, Inc., Logan, Utah) and penicillin (100 U/ml).

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M. leprae. Viable leprosy bacilli were freshly harvested for each experiment. A suspension of organisms was prepared from the footpad tissues of athymic mice (HSD athymic nu/nu/AF; Harlan Sprague-Dawley, Inc., Indianapolis, Ind.) that were infected with a rifampin-sensitive strain of *M. leprae* 13 to 16 months previously and harbored approximately 1×10^{10} to 5×10^{10} *M. leprae* per footpad at the time of harvest. Bacilli were recovered from the footpads by mincing and homogenization in a Ten Broeck tissue grinder (no. 357428; Wheaton Scientific, Millville, N.J.) in RPMI-0% FCS followed by low-speed centrifugation (50 × *g*) to remove tissue debris. The number of acid-fast bacilli in the supernatant medium was quantitated by using the techniques described by Shepard and McRae (22), and this suspension was diluted to the appropriate concentration in RPMI-20% FCS.

Formalin-killed *M. leprae* were prepared by incubating a suspension of freshly harvested organisms for 18 h in 10% buffered Formalin followed by copious washing with RPMI-0% FCS, enumeration, and suspension in RPMI-20% FCS.

Infection of macrophages. PC were diluted to 20×10^6/ml in RPMI-20% FCS (approximately 50% macrophages as determined by staining for nonspecific esterase activity), and 1.0-ml samples were placed in each of several 5-ml snap-cap culture tubes (no. 2054; Becton Dickinson and Co., Cockeysville, Md.). Medium (1.0 ml) was added to control tubes containing only PC, whereas a 1.0-ml suspension of *M. leprae*, diluted to 4×10^8/ml, was added to the remaining tubes (i.e., an infection ratio of approximately 40 bacteria per macrophage). These cultures were rotated for 1 h at 37°C to encourage phagocytosis and then diluted to 2×10^6 PC per ml of medium and plated in 5-ml aliquots per 25-cm^2 culture flask (no. 25100; Corning Glass Works, Corning, N.Y.). After an additional 2-h incubation period nonadherent cells and extracellular *M. leprae* were removed by washing, and the cultures were reincubated.

Rifampin. A stock solution of rifampin (Sigma Chemical Co., St. Louis, Mo.) was made by dissolving rifampin in absolute ethanol (5 mg/ml) and diluting in RPMI to a concentration of 100 μg/ml. The stock solution was diluted further in RPMI-20% FCS to the desired rifampin concentration.

Radiolabeled PA. Carbon-14 universally labeled PA ([U-^{14}C]PA; specific activity, 850 mCi/mmol) was obtained from

New England Nuclear Research Products (NEC 534; Berkeley, Calif.). At the appropriate time cultures were pulsed with 0.2 μCi of [U-^{14}C]PA per ml. When medium changes were required, the new medium also contained [U-^{14}C]PA in the same concentration.

Incorporation of [U-^{14}C]PA. After the appropriate interval, uptake of [U-^{14}C]PA was terminated by washing the cultures free of extracellular radiolabeled PA. The *M. leprae*-infected macrophages were removed with the aid of a rubber policeman and lysed by sonication (Sonifier cell disruptor, model W185D; Ultrasonics, Inc., Plainview, N.Y.) for 15 s at a setting of 75 W. *M. leprae* were quantitated as described above, and the sonicate was lyophilized. Total lipids were extracted by using the procedures of Folch et al. (6), and glycolipids were fractionated by the procedures described by Hunter and Brennan (9, 11). Briefly, lyophilized samples were extracted with 30 ml of chloroform-methanol (2:1) at 50°C for 18 h followed by 24 h of extraction with methanol. A biphasic wash was then made on the chloroform-methanol (2:1) extract by adding 6.0 ml of 0.7% KCl. The solution was gently shaken and allowed to separate into two distinct phases over 24 h. The upper aqueous phase was discarded, and the lower organic phase was pooled with the methanol extract and dried under vacuum in a rotary evaporator. Total lipids were then solubilized in 0.5 ml of chloroform, loaded on a column of Florisil-silicic acid (Sigma F-7752; 2:1; bed volume, 1.5 ml), and fractionated by sequential elution with 2 bed volumes each of chloroform, 2% methanol in chloroform, and 5% methanol in chloroform. The chloroform extract containing neutral lipids was discarded, and the methanol-chloroform extracts were pooled, dried, and dissolved in 200 μl of chloroform. Then 10 μl of this extract was spotted on a thin-layer chromatography (TLC) plate (T-7520; Sigma), and a 5-μl sample of authentic PGL-I (200 μg/ml; supplied by Patrick Brennan, Colorado State University, Fort Collins, Colo.) was added to serve as a marker. The TLC plates were developed in a solvent system of chloroform-methanol-water (90:10:1), and the glycolipid spot was stained with 0.1% orcinol in 40% sulfuric acid. The stained PGL-I spot was scraped from the TLC plate into scintillation fluid, and the amount of [U-^{14}C]PA incorporated into PGL-I was quantitated in a liquid scintillation counter. Values were then normalized to counts per minute per 10^8 *M. leprae*.

Identification of PGL-I. To identify labeled PGL-I a modification of the technique described by Young et al. (23) was used. The labeled spot corresponding to PGL-I on the TLC plate, was scraped, extracted with chloroform-methanol (2:1), and suspended in 100 μl of hexane. A 10-μl sample was spotted on a polysulfone membrane strip (supplied by Gelman Sciences as Tuffryn membrane filters HT-200; 0.2-μm pore size), and standard PGL-I in hexane was spotted alongside. The polysulfone membrane was developed in a solvent system of hexane-ether (80:20) for 30 min, and the strips were dried and blocked for 30 min in 5% bovine serum albumin in phosphate-buffered saline (PBS) (pH 7.2). The strips were then treated with immunoglobulin G monoclonal antibody to PGL-I, Mab 46.5 (15) (supplied by Barry Bloom, Albert Einstein College of Medicine, New York, N.Y.), diluted 1:2,500 in 5% bovine serum albumin in PBS for 1 h at room temperature, washed in PBS for 30 min, reacted with peroxidase-conjugated goat anti-mouse immunoglobulin G (Dako Corp., Santa Barbara, Calif.) at a dilution of 1:500 for 1 h, and washed in PBS for 30 min. The polysulfone membrane strips were then developed in the substrate dye reagent containing 60 mg of HRP (4 chloro-1 napthol; supplied by Bio-Rad Laboratories, Richmond, Calif.) and 600 μl of H_2O_2 (3%) in 100 ml of Tris hydrochloride buffer (pH 7.5). The reaction was stopped after 15 min with 5% acetic acid. PGL-I was visualized as an intense blue spot on a white background. The strip containing the radioactive PGL-I was cut uniformly in 0.50-cm sections, and the radioactivity was quantitated in each individual section.

TABLE 1. Incorporation of [U-^{14}C]PA into PGL-I of live and Formalin-killed *M. leprae* in macrophages[a]

Expt no.	Incubation (days)	cpm/10^8 *M. leprae*	
		Live	Killed
1	8	3,200	320
	15	8,920	355
2	8	8,480	657
	18	25,080	1,260
3	8	10,100	ND[b]
	16	15,600	1,840

[a] [U-^{14}C]PA (1.0 μCi) was added to each culture flask at the time of infection with *M. leprae* and at subsequent changes of medium (every 5 days).
[b] ND, Not done.

Statistics. The Student *t* test was used to test the significance of experimental results.

RESULTS

Incorporation of [U-^{14}C]PA by *M. leprae*. Shown in Table 1 are the results of three experiments which compare the incorporation of labeled [U-^{14}C]PA into the glycolipid fraction cochromatographing with authentic PGL-I on the TLC plates from macrophages infected with live *M. leprae* or with Formalin-killed *M. leprae* at two time intervals. In these initial studies [U-^{14}C]PA was added to all cultures at 0 h; sample cultures were terminated at 8 days and either 15, 16, or 18 days later. The radioactivity associated with the glycolipid fractions from macrophages infected with both live and killed *M. leprae* was virtually the same. However, when the TLC of the glycolipid fraction was carried out, with analysis limited solely to the spot corresponding to the R_f value of authentic PGL-I, label was found only in extracts from live *M. leprae*-infected macrophages. The percent incorporation of labeled PA into the PGL-I fraction varied depending on experimental conditions (i.e., different *M. leprae* inocula and time of incubation) but did not exceed 1% (range, 0.4 to 0.6%). There was little or no incorporation of PA into this fraction when Formalin-killed *M. leprae* or noninfected macrophage cultures were used. Two time intervals were employed, and in each of the three experiments, increased incubation periods resulted in increased incorporation of PA into the spot with the same R_f value as PGL-I.

To establish the identity of the labeled spots, the area in the silica gel with the same R_f value as PGL-I was removed from the TLC plate, extracted, chromatographed on polysulfone membranes, and interacted with monoclonal anti-PGL-I. Clearly, the unknown labeled spot appeared to be identical to PGL-I (Fig. 1), and virtually all of the radioactivity was located in the PGL-I spot.

Effects of rifampin. Although Formalin-killed *M. leprae* was shown to be nonviable as defined by the inability to incorporate [U-^{14}C]PA into PGL-I, it was important to determine if killing or inhibition of *M. leprae* within the macrophage could also be detected with this assay. The results of an experiment designed to test the effects of the bactericidal drug rifampin on incorporation of PA into the PGL-I of *M. leprae* are shown (Fig. 2). In these initial studies

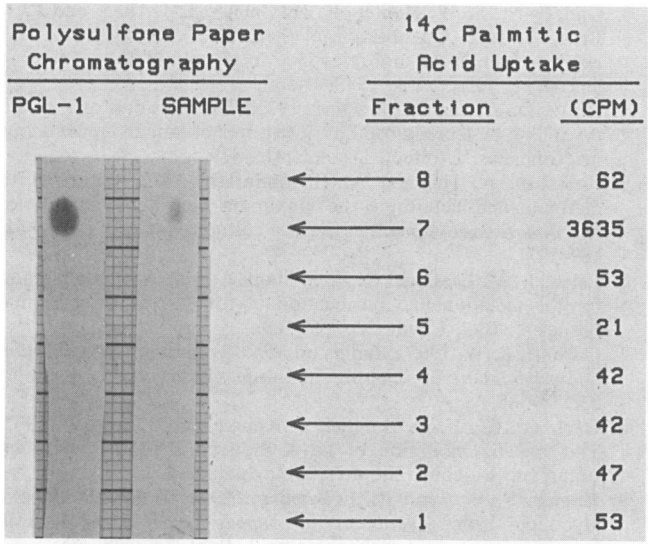

FIG. 1. Polysulfone paper chromatography of authentic PGL-I and [U-^{14}C]-labeled glycolipid from *M. leprae*-infected macrophages. Both strips were stained with peroxidase-labeled anti-PGL-I immunoglobulin G monoclonal antibody 46.7. Shown are the counts per minute (cpm) of [U-^{14}C]PA found incorporated into the different fractions of the chromatographed sample from *M. leprae*-infected macrophages shown in lane 2 (SAMPLE). Lane 1, authentic PGL-I.

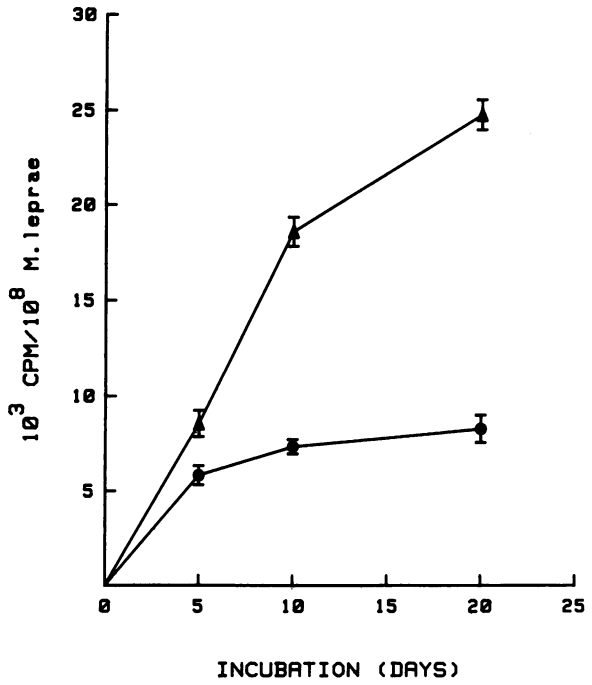

FIG. 2. Effects of rifampin on incorporation of [U-^{14}C]PA into PGL-I of *M. leprae* in macrophages. Each point represents the mean counts per minute (cpm) and standard deviation of duplicate samples from each group. Rifampin and [U-^{14}C]PA were added to the cultures at the time of infection with *M. leprae* (0 h). Sample cultures were analyzed at 5, 10, and 20 days. ▲, Control; ●, 2.0 μg of rifampin per ml.

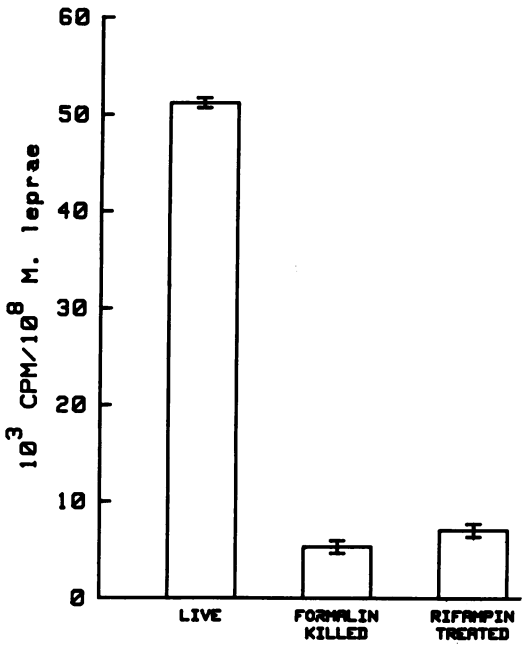

FIG. 3. Effects of rifampin on incorporation of [U-^{14}C]PA into PGL-I of *M. leprae* in macrophages. Shown is the mean counts per minute plus or minus standard deviation of triplicate samples from each group. [U-^{14}C]PA was added to the cultures at 4 days after infection with live or Formalin-killed *M. leprae*, and the cultures were analyzed at 14 days. Rifampin (2 μg/ml) was added to the treated group from the onset of culture.

2.0 μg of rifampin per ml was used. Rifampin and [U-^{14}C]PA were added at the beginning of the experiment, and the cultures were terminated 5, 10, or 20 days later. There was no apparent effect on PA incorporation within the first 5 days of culture. However, whereas the incorporation of PA into *M. leprae* PGL-I increased significantly over the subsequent 10 and 20 days of culture in untreated macrophages, there was no further incorporation of label in cultures containing rifampin ($P < 0.005$ at both 10 and 20 days; N = 2).

In the experiment depicted in Fig. 3 rifampin was added to the appropriate cultures at 0 h; however, in contrast to the procedure described above the addition of [U-^{14}C]PA was delayed until day 4 of culture to amplify the effects of rifampin treatment. The cultures were terminated and assayed for PGL-I synthesis on day 14. Live *M. leprae* incorporated over 10 times the amount of labeled PA into the PGL-I fraction than did Formalin-killed or rifampin-treated *M. leprae* ($P < 0.001$; N = 3).

DISCUSSION

Since *M. leprae* does not grow in vitro in any known culture medium, the animal and tissue culture systems that allow metabolism or multiplication of the organism have unusual importance for drug assays or for detecting viability. There have been conflicting reports on the behavior of *M. leprae* in tissue culture (2, 7, 14), but most workers would agree that successful multiplication of *M. leprae* in tissue culture has not been attained (5, 20). However, there is evidence of metabolism by *M. leprae* in vitro as determined by the incorporation of radiolabeled thymidine or leucine into DNA or protein fractions of *M. leprae* in experimentally infected cells (4, 18, 19).

The present work measures an increase in an *M. leprae*-specific product in a system in which indiscriminate labeling of host cell constituents is not a problem. Large quantities of PGL-I have been detected in the infected tissues of lepromatous leprosy patients, in infected armadillos, and in the serum of lepromatous patients (3, 24), implying that PGL-I synthesis represents a major metabolic pathway of *M. leprae*. Effective chemotherapy has been observed to lower the concentration of PGL-I in infected tissues and blood (24), further suggesting that synthesis of PGL-I might be a quantifiable indicator of the overall viability of the leprosy bacillus.

Macrophages maintain the viability of *M. leprae* for prolonged periods in vivo. The present study shows that macrophages provide suitable conditions for studying the metabolism of *M. leprae* in vitro for at least 3 weeks.

In addition to providing a method of quantitating *M. leprae* metabolism in vitro, these preliminary studies demonstrated a clear difference in the incorporation of [U-^{14}C]PA into the PGL-I fraction of live and killed *M. leprae*, suggesting this technique could be used to measure the effects of antileprosy chemotherapeutic agents. The present studies also demonstrate the ability of rifampin to almost completely block the incorporation of PA into PGL-I. Because rifampin exerts its bactericidal effect by binding to DNA-dependent RNA polymerase, thereby inhibiting RNA formation and subsequent protein synthesis (8, 17), it is unlikely that rifampin acts directly on the pathways leading to PGL-I synthesis in *M. leprae*.

These preliminary observations show that PGL-I synthesis can be used to quantitate the metabolism of *M. leprae*, differentiating it from host cell metabolism. It is important to emphasize that this assay provides quantitative evidence that is only consistent with viability of the leprosy bacillus. Incorporation of [U-^{14}C]PA into *M. leprae* PGL-I is a metabolic event distinct from host cell metabolism, but inhibition of PGL-I synthesis does not necessarily mean the bacilli have been killed. The method seems sufficiently sensitive and reproducible to suggest further evaluation as a means of screening antileprosy drugs in vitro, monitoring the results of attempts to cultivate *M. leprae*, and exploring the role of host mononuclear phagocytes in resistance to the leprosy bacillus. Further studies correlating the inhibition of PGL-I synthesis with the actual viability of *M. leprae* by the standard mouse footpad technique are needed before one could unequivocally suggest this technique as a means for monitoring the viability of *M. leprae*.

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LITERATURE CITED

1. **Brennan, P. J., and W. W. Barrow.** 1981. Evidence for species specific lipid antigens in *Mycobacterium leprae*. Int. J. Lepr. **48**:382–387.
2. **Chang, Y. T., and R. L. Neikerk.** 1965. *Mycobacterium lepraemurium* and *Mycobacterium leprae* in cultures of mouse peritoneal macrophages. Int. J. Lepr. **33**:586–598.
3. **Cho, S. N., S. W. Hunter, R. H. Gelber, T. H. Rea, and P. J. Brennan.** 1986. Quantitation of the phenolic glycolipid of *Mycobacterium leprae* and relevance to glycolipid antigenemia in leprosy. J. Infect. Dis. **153**:560–569.
4. **Drutz, D. J., and M. J. Kline.** 1972. Incorporation of tritiated thymidine by leprosy bacillus in cultures of human lepromatous macrophages. J. Infect. Dis. **125**:416–419.
5. **Fieldsteel, A. H., and A. H. McIntosh.** 1972. Attempts to cultivate and determine the maximum period of viability of *Mycobacterium leprae* in tissue culture. Int. J. Lepr. **52**: 189–197.
6. **Folch, J., M. Lees, and G. H. S. Stanley.** 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. **226**:497–509.
7. **Garbutt, E. W.** 1965. Studies on *Mycobacterium lepraemurium* and *Mycobacterium leprae* in tissue cultures. Int. J. Lepr. **33**:578–585.
8. **Hartman, G., K. O. Hanikel, F. Knusel, and T. Nuesch.** 1967. The specific inhibition of DNA induced RNA synthesis by rifamycin. Biochim. Biophys. Acta **145**:843–851.
9. **Hunter, S. W., and P. J. Brennan.** 1981. A novel phenolic glycolipid from *Mycobacterium leprae* possibly involved in immunogenicity and pathogenicity. J. Bact. **147**:728–735.
10. **Hunter, S. W., T. Fujiwara, and P. J. Brennan.** 1982. Structure and antigenicity of the major specific glycolipid antigen of *Mycobacterium leprae*. J. Biol. Chem. **257**:15072–15078.
11. **Hunter, S. W., C. Stewart, and P. J. Brennan.** 1985. Purification of phenolic glycolipid-I from armadillo and human sources. Int. J. Lepr. **53**:484–486.
12. **Krahenbuhl, J. L., and J. S. Remington.** 1974. The role of activated macrophages in specific and non-specific cytostasis of tumor cells. J. Immunol. **113**:507–516.
13. **Levy, L.** 1976. Studies of the mouse footpad technique for cultivation of *Mycobacterium leprae*. Lepr. Rev. **47**:103–106.
14. **McCrae, D. H., and C. C. Shepard.** 1970. Attempts to cultivate *Mycobacterium leprae* in tissue culture. Int. J. Lepr. **38**: 342–343.
15. **Mehra, V., P. J. Brennan, E. Rada, J. Convit, and B. R. Bloom.** 1984. Lymphocyte suppression in leprosy induced by unique *M. leprae* glycolipid. Nature (London) **308**:194–196.
16. **Mukherjee, R., Y. Mistry, N. H. Antia, N. Klein, and N. Vemuri.** 1985. Incorporation of ^{14}C acetate into the specific phenolic glycolipid of *M. leprae*, maintained within cultured cells. IRCS Med. Sci. **13**:203–204.
17. **Pallanza, R., V. Arioli, S. Furesz, and G. Bolozonie.** 1967. Rifampicin a new rifamycin. Arzneim. Forsch. **17**:529–531.
18. **Prasad, H. K., and R. C. Hastings.** 1985. Alternate radiolabeled markers for detecting metabolic activity of *Mycobacterium leprae* residing in murine macrophages. J. Clin. Microbiol. **21**:861–864.
19. **Satish, M., and I. Nath.** 1981. The uptake of ^{3}H thymidine in *Mycobacterium leprae* inoculated mouse macrophages as a rapid indicator of bacterial viability. Int. J. Lepr. **49**:187–193.
20. **Sharp, A. K., and D. K. Bannerji.** 1984. Attempts at cultivation of *Mycobacterium leprae* in macrophages from susceptible animal hosts. Int. J. Lepr. **52**:189–197.
21. **Shepard, C. C.** 1960. The experimental disease that follows the injection of human leprosy bacillus into footpads of mice. J. Exp. Med. **112**:445–454.
22. **Shepard, C. C., and D. H. McCrae.** 1968. A method for counting acid fast bacilli. Int. J. Lepr. **36**:78–82.
23. **Young, D. B., M. J. Fohu, and T. M. Buchanan.** 1985. Use of a polysulfone membrane support for immunochemical analysis of a glycolipid from *Mycobacterium leprae*. J. Immunol. Methods **79**:205–211.
24. **Young, D. B., J. P. Harnisch, J. Knight, and T. M. Buchanan.** 1985. Detection of PGL-I in serum of patients with lepromatous leprosy. J. Infect. Dis. **152**:1078–1087.